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RESEARCH PAPER

N-Trimethyl Chitosan Chloride: Optimum Degree of Quaternization for Drug Absorption Enhancement Across Epithelial Cells

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ABSTRACT

N-trimethyl chitosan chloride (TMC) is a polycation that enhances drug transport across epithelia by opening tight junctions. The degree of quaternization of TMC determines the number of positive charges available on the molecule for interactions with the negatively charged sites on the epithelial membrane and thereby influences its drug absorption-enhancing properties. The effects of six different TMC polymers (degree of quarternization between 12% and 59%) on the transepithelial electrical resistance (TEER) of Caco-2 cell monolayers and on the transport of hydrophilic and macromolecular model compounds across Caco-2 cells were determined. All the TMC polymers were able to decrease the TEER markedly in a slightly acidic environment (pH 6.2). However, only TMC polymers with higher degrees of quaternization (>22%) were able to reduce the TEER in a neutral environment (pH 7.4). The maximum reduction in TEER ($47.3 \pm 6.0\%$ at a concentration of 0.5% w/v and pH 7.4) was reached with TMC with a degree of quaternization of 48%, and this effect did not increase further with higher degrees of quaternization of TMC. In agreement with the TEER results, the transport of model compounds across Caco-2 cell monolayers increased with an increase in the degree of quaternization of TMC. However, the transport reached a maximum for TMC with a degree of quaternization of 48% (25.3% of the initial dose for [^{14}C]mannitol and 15.2% of the initial dose for [^{14}C]PEG 4000), and this effect did not increase further with higher degrees of quaternization of TMC. Therefore, the increase in the effects of TMC on intestinal epithelia did not directly correlate up to the maximum quaternization degree of this polymer, but reached an optimum value already at an intermediate degree of quaternization (ca. 48%).

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INTRODUCTION

N-trimethyl chitosan chloride (TMC), a partially quaternized derivative of chitosan with superior water solubility, can be synthesized by reductive methylation of the amino groups in the C-2 position of chitosan during a reaction with methyl iodide in the presence of a strong base.[1] The degree of quaternization of TMC can be controlled by the number and duration of the reaction steps used during the synthesis process.[2,3] Complete quaternization of the starting polymer, chitosan, is not possible because of the presence of acetyl groups and steric effects caused by the attached methyl groups.[3] TMC has the ability to enhance the absorption of hydrophilic and macromolecular drugs across mucosal epithelia. TMC is also effective in neutral environments, where chitosan salts precipitate from solution, rendering them ineffective as absorption enhancers at neutral pH values.[4–6]

Several studies have shown that chitosan acts as an absorption enhancer by opening the tight junctions between adjacent epithelial cells through interactions between the protonated (positively charged) amino groups on the C-2 position of this compound and the negatively charged sites on the cell membrane and/or in the tight junctions.[7,8] It has been proposed that TMC acts by the same mechanism as chitosan to enhance the paracellular absorption of drugs.[4,5,9] The charge density of TMC, as determined by the degree of quaternization, plays an important role in the absorption-enhancing properties of this polymer, especially in neutral and alkaline environments. It was clearly shown that the transepithelial electrical resistance (TEER)-reducing ability and absorption-enhancing properties of TMC increase with an increase in the degree of quaternization in a neutral environment. This was explained by a higher number of fixed positive charges (quaternized amino groups) available on TMC molecules with higher degrees of quaternization for interactions with the negative sites on the cell membranes and/or in the tight junctions.[10–12] However, this may only apply to TMC polymers up to an optimum degree of quaternization, because of possible changes in molecular flexibility of the TMC polymer chain. These changes may be caused by electrostatic repulsive forces between the positively charged amino groups on the repeating units. Furthermore, steric effects caused by the attached methyl groups may also hide the positive charges on the amino groups.

Although it was shown that TMC with a high degree of quaternization (= 60%) was more effective in enhancing the transport of a hydrophilic model compound across Caco-2 cell monolayers, compared to TMC with lower degrees of quaternization (= 12% and 40%),[12,13] the hypothesis was investigated in this study that TMC with the highest possible degree of quaternization (ca. 60%) may not be the optimum degree of quaternization for absorption enhancement by TMC in a neutral environment due to the steric effects caused by the attached methyl groups and flexibility of the molecule.

Absorption enhancers, like any other pharmaceutical additive, can adversely affect epithelial cells at the site of contact, or systemic toxicity may occur when it is absorbed into the systemic circulation. Irritation of mucosal tissues by absorption enhancers, the extent of damage in the mucosal cells, and the rate at which such damage recovers are three of the most pressing safety concerns associated with the use of these substances.[14] Local effects on epithelial integrity and functionality are usually determined with techniques involving microscopy, but techniques such as measurement of the ciliary beat frequency (CBF) of ciliated epithelial cells are also sensitive and useful methods to determine local epithelial toxicity.[15] The epithelium of the upper respiratory tract is covered by many hair-like cilia beating in a coordinated manner within the periciliary fluid beneath a layer of viscoelastic mucus, thereby moving particles toward the pharynx where they are swallowed. This mucociliary clearance is an important defense mechanism of the human body against inhaled dust, allergens, and microorganisms. A decreased motility may be predictive of a local toxic influence on the epithelial mucosal cells. However, if the rate of clearance is slightly decreased or halted in a reversible manner, such retardation might be useful and could result in an increased contact time between the drug and nasal mucosa, thereby increasing the bioavailability.[16]

The aim of this study was to identify the optimum degree of quaternization of TMC for absorp-

tion enhancement of hydrophilic and macromolecular compounds across intestinal epithelial cell monolayers in a neutral environment. The effect of TMC on the CBF of human nasal epithelia was determined as an indication of local toxicity on mucosal epithelial cells.

MATERIALS AND METHODS

Synthesis and Characterization of TMC Polymers

The synthesis method previously described by Domard et al.,[1] Sieval et al.,[2] and Hamman and Kotzé[3] was used to prepare six different TMC polymers with varying degrees of quaternization. The reaction was repeated several times under the same conditions to synthesize TMC polymers with varying degrees of quaternization. The starting polymer for all the synthesized TMC products was 93% deacetylated chitosan, except for TMC-12, in which 80% deacetylated chitosan was used (both obtained from Pronova Biopolymer, Drammen, Norway). The use of 80% deacetylated chitosan in the synthesis of TMC can be explained by the degree of quaternization of TMC obtained from 93% deacetylated chitosan was already above 20% for a one-step reaction.[3]

The TMC polymers were characterized by means of proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). Samples (10 mg) of each synthesized polymer were dissolved in D_2O in a NMR tube, and the solutions were measured in a 600 MHz DMX Brucker apparatus (Karlsruhe, Germany). The degrees of quaternization were calculated using the following formula[12]:

$$DQ(\%) = \left[\left(\int TM \Big/ \int H\right) \times \frac{1}{9}\right] \times 100$$

where: DQ (%) = degree of quaternization as a percentage, $\int TM$ = integral of the trimethyl amino group (quaternary amino group) peak at 3.3 ppm, and $\int H$ = integral of the ^{1}H peaks at 4.7–5.7 ppm.

Culturing of Caco-2 Cell Monolayers

Caco-2 cells (American Type Culture Collection, Manassas, Virginia, passages 21–33) were seeded on tissue culture-treated polycarbonate filters (area = 0.33 cm^2 and 4.70 cm^2) in Costar Transwell 24-well and 6-well plates (Corning Costar Corporation, USA), respectively, at a density of 1.77×10^4 cells/mL to determine the effect of the synthesized TMC polymers on TEER of the cell monolayers (24-well plates), and to measure the transport of hydrophilic and macromolecular model compounds across the cell monolayers (6-well plates). The culture medium consisted of Dulbecco's Modified Eagles Medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Delta Bioproducts, Kempton Park, South Africa), 1% non-essential amino acids (BioWhittaker), and 1% pen/-strep fungizone mixture (10,000 units penicillin/mL, 10,000 μg streptomycin/mL, and 25 μg fungizone/mL; BioWhittaker). The culture medium was added to both the apical (200 μL for the 24-well plates and 2.5 mL for the 6-well plates) and basolateral (1 mL for the 24-well plates and 2.5 mL for the 6-well plates) compartments of the filters. The culture medium was changed every second day under aseptic conditions, and the cell cultures were kept in an incubator at a temperature of 37°C in an atmosphere of 95% air and 5% CO_2. The confluent cell monolayers were used for TEER or transport measurements between 19–23 days postseeding of the cells onto the filters.

TEER Measurements

The effect of the TMC polymers (0.25 and 0.5% w/v) on the TEER of Caco-2 cell monolayers was investigated at two pH values (6.2 and 7.4). The polymer solutions were prepared in serum-free DMEM, and the pH was adjusted to 6.2 with 0.1 M HCl and to 7.4 with 0.1 M NaOH, respectively. The TEER was measured using a Millicell ERS (Millipore) meter connected to chopstick electrodes. TEER measurements at time intervals of 20 min started 1 hr before incubation on the apical side of the cells with the different TMC solutions and proceeded for 2 hr after incubation. The reversibility of the effect of TMC on the TEER was measured by removing the TMC solutions from the apical side of the cells after a period of 2 hr, and it was replaced by serum-free DMEM, whereas the TEER was measured for another 2 hr. Control experiments were performed under the same conditions, but without dissolved TMC polymers. All the experiments were done in triplicate at a temperature of 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Transport of [^{14}C]Mannitol

A volume of 2.5 mL of the TMC solutions (0.25 and 0.5% w/v) in serum-free DMEM at pH 7.4 containing 1 μL/mL [^{14}C]mannitol (Amersham Life Sciences, Little Chalfort, UK; specific radioactivity 200 mCi/mL, mol. wt. = 182.2) was added on the apical side of the Caco-2 cell monolayers. The medium in the acceptor compartment was DMEM buffered at a pH of 7.4 with 25 mM HEPES [*n*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid)] (BioWhittaker). Samples of 200 μL were taken every 20 min from the basolateral side for 4 hr after incubation of the solutions on the apical side of the monolayers. The samples withdrawn from the basolateral side were replaced with an equal volume of DMEM containing HEPES. Control experiments were run using solutions of the radioactive marker without the dissolved TMC polymers. All experiments were performed in triplicate in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The radioactivity present in the samples was determined after adding 3 mL of scintillation cocktail (Ready Gel, Beckman, Fullerton, CA) in a liquid scintillation counter (Beckman LS 3801). Results obtained from these samples were corrected for dilution and expressed as the cumulative transport (percentage of initial dose) at time t. Apparent permeability coefficients (P_{app}) for [^{14}C]mannitol were calculated according to the following equation:

$$P_{app} = dQ/dt\{1/(A \cdot 60 \cdot C_0)\}$$

where P_{app} = apparent permeability coefficient ($cm \cdot sec^{-1}$), dQ/dt = permeability rate (amount permeated per minute), A = diffusion area of the monolayer (cm^2), and C_0 = initial concentration of the marker molecule. Transport enhancement ratios (R) were calculated from the P_{app} values by using the following equation:

$$R = P_{app}(\text{sample})/P_{app}(\text{control})$$

Transport of [^{14}C]Polyethylene Glycol 4000

A volume of 2.5 mL of the TMC solutions (0.125 and 0.5% w/v) in serum-free DMEM at pH 7.4 containing 4 μL/mL [^{14}C]polyethylene glycol 4000 (PEG) (Amersham Life Sciences; specific radioactivity 50 mCi/mL, mol. wt. = 4,000) was added on the apical side of the Caco-2 cell monolayers. Transport of this macromolecular model compound was determined as described for [^{14}C]mannitol.

Data Analysis and Statistical Evaluation

Averages and standard deviations were calculated for the effects of each TMC polymer on the TEER and transport of the model compounds. Statistical differences between the effects of the different polymers were evaluated with the method of Duncan (analysis of variance; SAS Institute, Inc., Cary, NC) on the mean values obtained from each experiment. Results were considered statistically different with $p < 0.05$.

CBF Measurements

Experiments on human nasal epithelial cells were approved by the ethical committee of Potchefstroom University for Christian Higher Education (Potchefstroom, South Africa) and were performed according to the regulations set by the ethical committee. The nostril of a healthy human subject was inspected via a Welch Allyn Diagnostic set. A nylon nasal cytology brush (Hobbs Medical, Inc., South Africa) was inserted through the diagnostic set (window removed), and nasal epithelial cells were harvested from the inferior turbinate. These epithelial cells were suspended immediately in 5 mL of prewarmed (37°C) DMEM, and this suspension was kept at a temperature of 37°C. TMC solutions were prepared in serum-free DMEM in a concentration of 1.0% w/v. A sample from the cell suspension was used for the control, and a 0.5-mL cell suspension was added to 0.5 mL of each TMC solution to prepare test solutions with a final concentration of 0.5% w/v.

Samples from the control cell suspension and test solutions were transferred to a pre-prepared area surrounded by high-vacuum grease on glass microscope slides. A cover slip was gently pressed onto the high-vacuum grease to seal the preparation. This ensures that the cover slip does not press directly onto the sample of human nasal epithelium and interfere with ciliary beat activity. The sealed slide preparation was transferred to the hot stage (37°C) of a microscope (Olympus BH2, Japan) equipped with a video camera (Panasonic, Japan) connected to a video monitor (Panasonic, Japan). The television

signals relating to differences in light intensity resulting from ciliary motion was determined by a PCX video digitizer card (programmed by the CSIR, South Africa) and computed directly into units of Hertz (Hz). The CBF (Hz) of the nasal cells in the sample was measured every 15 min (10 readings per time interval) over a period of 1 hr. The mean CBF (percentage of initial value) was plotted as a function of time for all the TMC polymers.[18]

RESULTS AND DISCUSSION

Synthesis and Characterization of TMC Polymers

The number of reaction steps used in the synthesis of each TMC polymer and the calculated degrees of quaternization of the products are shown in Table 1. The degree of quaternization of the TMC polymers increased with an increase in the number of reaction steps, and the highest degree of quaternization for TMC was 59.2%.

Effect of TMC on the TEER of Caco-2 Cell Monolayers

The effect of the TMC polymers in a concentration of 0.5% w/v at pH 6.2 on the TEER of the Caco-2 cell monolayers is depicted in Fig. 1. Reduction in the TEER of the Caco-2 cell monolayers, 2 hr after incubation with the TMC polymers at a concentrations of 0.5% w/v and 0.25% w/v is shown in Table 2.

From Fig. 1, it is clear that TMC with a degree of quaternization as low as 12% already caused a pronounced reduction in TEER, but this effect of TMC-12 is still relatively low compared with the other TMC polymers with higher degrees of quaternization. Although the effect on the TEER increases with an increase in the degree of quaternization, only relatively small differences between the effects on the TEER at pH 6.2 was observed for the TMC polymers with higher degrees of quaternization ($\geq$22%). This indicates that the degree of quaternization of TMC does not play an important role in the opening of tight junctions of Caco-2 cell monolayers in an acidic environment (pH 6.2). Additional protonation of the monomethylated and dimethylated amino groups is possible at this pH value to form quaternized amino groups. More positively charged, quaternized amino groups are therefore available on the TMC molecules to interact with the negatively charged sites on the cell membranes and within the tight junctions, which influences the extent of tight junction opening and the number of tight junctions that are opened.

The effect of the TMC polymers in a concentration of 0.5% w/v at pH 7.4 on the TEER of the Caco-2 cell monolayers is depicted in Fig. 2. Reduction in the TEER of the Caco-2 cell monolayers, 2 hr after incubation with the TMC polymers at concentrations of 0.25 and 0.5% w/v, is shown in Table 2.

Only TMC polymers with higher degrees of quaternization ($>$22%) were able to decrease the TEER of Caco-2 cell monolayers at pH 7.4. The effect of the TMC polymers on the TEER of Caco-2 cell monolayers increased markedly with an increase in the degree of quaternization of TMC, but reached a plateau at a quaternization degree of 48% with no further increases in the reduction of TEER. No significant differences in the reduction of the TEER were found between the highly quaternized TMC polymers (TMC-48, TMC-55, and TMC-59).

Table 1. Number of reaction steps used to synthesize the different TMC polymers and their calculated degrees of quaternization.

TMC polymer	No. of reaction steps	Degree of quaternization (%)
TMC-12	1 (with 80% deacetylated chitosan)	12.3
TMC-22	1 (with 93% deacetylated chitosan)	22.1
TMC-36	2 (with 93% deacetylated chitosan)	36.3
TMC-48	3 (with 93% deacetylated chitosan)	48.0
TMC-55	3 (with 93% deacetylated chitosan)	55.2
TMC-59	4 (with 93% deacetylated chitosan)	59.2

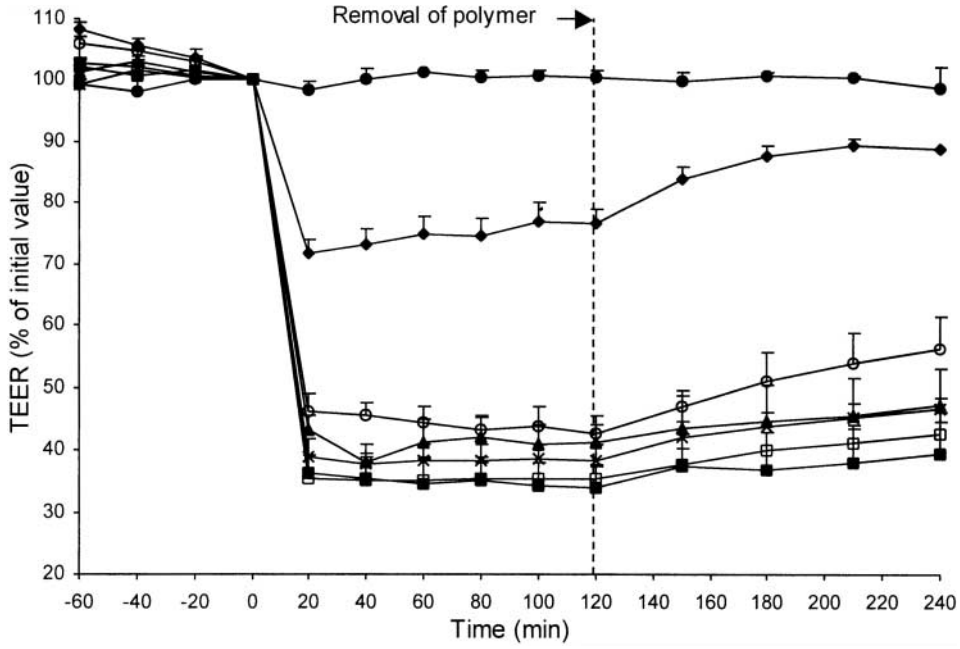

Figure 1. Effect of TMC polymers (0.5% w/v) at pH 6.2 on the TEER of Caco-2 cell monolayers. Key: control (●), TMC-12 (◆), TMC-22 (○), TMC-36 (▲), TMC-48 (■), TMC-55 (×), and TMC-59 (□). Each point represents the mean ± SD of three values.

Table 2. Percentage of reduction in TEER of Caco-2 cell monolayers, 2 hr after incubation with the TMC polymers at pH 6.2 and 7.4, respectively.

	pH 6.2		pH 7.4	
TMC polymer	0.25% w/v	0.5% w/v	0.25% w/v	0.5% w/v
Control	0.0 ± 1.0^{D}	0.0 ± 1.0^{D}	0.0 ± 4.2^{D}	0.0 ± 4.2^{C}
TMC-12	19.9 ± 0.6^{C}	23.3 ± 2.1^{C}	1.2 ± 4.0^{D}	0.1 ± 1.7^{C}
TMC-22	46.4 ± 2.5^{B}	57.3 ± 2.8^{B}	19.5 ± 2.5^{C}	24.2 ± 5.6^{B}
TMC-36	59.8 ± 2.9^{A}	58.8 ± 2.8^{B}	37.3 ± 4.3^{B}	41.5 ± 3.0^{A}
TMC-48	61.4 ± 1.8^{A}	66.2 ± 3.6^{A}	49.4 ± 4.5^{A}	47.3 ± 6.0^{A}
TMC-55	57.0 ± 2.0^{A}	$61.7 \pm 2.5^{A,B}$	41.1 ± 1.9^{B}	41.6 ± 5.1^{A}
TMC-59	59.5 ± 3.4^{A}	64.5 ± 2.3^{A}	50.4 ± 3.1^{A}	46.8 ± 3.0^{A}

Each value represents the mean ± SD of three experiments. Values marked with the same letter (A, B, C, D) are not significantly different.

Correlation between the degree of quaternization of TMC and the effect on the TEER can be explained by the fact that TMC polymers with higher degrees of quaternization have a higher number of fixed quaternized amino groups available for interactions with the negatively charged sites on the cell membranes and in the tight junctions. The number of these interactions determines the extent of the opening of the tight junctions and/or the number of tight junctions that are opened. In contrast with the results at pH 6.2, it is clear that the charge density on the TMC molecule, as determined by the degree of quaternization, plays an important role on the opening of the tight junctions of the Caco-2 cell monolayers at pH 7.4, where no additional protonation of the amino groups could take place. A maximum effect on the TEER of the Caco-2 cell monolayers was reached with TMC-48, probably

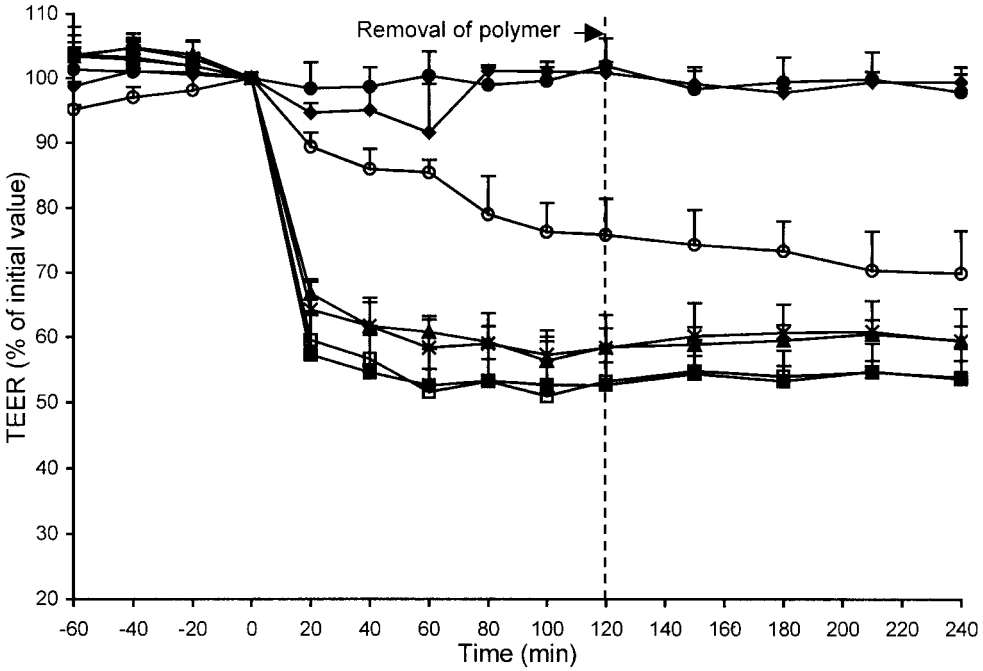

Figure 2. Effect of TMC polymers (0.5% w/v) at pH 7.4 on the TEER of Caco-2 cell monolayers. Key: control (●), TMC-12 (◆), TMC-22 (○), TMC-36 (▲), TMC-48 (■), TMC-55 (×), and TMC-59 (□). Each point represents the mean ± SD of three values.

because of a favorable conformation and chain flexibility of the molecule at this degree of quaternization. Possible steric effects caused by the attached methyl groups could probably start to play a role at higher degrees of quaternization.

A very slight recovery of the TEER of the Caco-2 cell monolayers toward the initial TEER values can be observed after removal of the TMC solutions. As a result of the high viscosity and mucoadhesive characteristics of TMC, it is most unlikely that all the polymer solution could be removed from the monolayers without damaging the cells. Therefore, the reversibility measured was only gradual and does not necessarily indicate damage to the membranes and tight junctions of the Caco-2 cells.

Effect of TMC on the Transport of [^{14}C]Mannitol

The cumulative transport of [^{14}C]mannitol (percentage of initial dose) across the Caco-2 cell monolayers in the presence of the TMC polymers (0.5% w/v) at pH 7.4 is depicted in Fig. 3. The cumulative amount of [^{14}C]mannitol (percentage of initial dose) transported across the Caco-2 cell monolayers, 4 hr after incubation with the TMC solutions (0.25 and 0.5% w/v), the calculated apparent permeability coefficients (P_{app}) and transport enhancement ratios (R) are given in Table 3. The values in Table 3 represent a 5.3-fold (TMC-48), 5.1-fold (TMC-36), 4.5-fold (TMC-55), 4.0-fold (TMC-59), 3.6-fold (TMC-12), and 3.0-fold (TMC-22) increase in the transport at a concentration of 0.5% w/v and a 6.0-fold (TMC-48), 5.5-fold (TMC-36), 4.9-fold (TMC-59), 4.0-fold (TMC-55), 3.3-fold (TMC-12), and 1.9-fold (TMC-22) increase in the transport at a concentration of 0.25% w/v.

A clear correlation between the degree of quaternization of TMC and the transport enhancement of [^{14}C]mannitol across Caco-2 cell monolayers could not be established in this experiment. In general, the effect of TMC on the transport of [^{14}C]mannitol increased, with an increase in the degree of quaternization until a maximum effect was reached with TMC-48. This effect did not increase further for the TMC polymers with higher degrees of quaternization (55% and 59%). The transport enhancement of

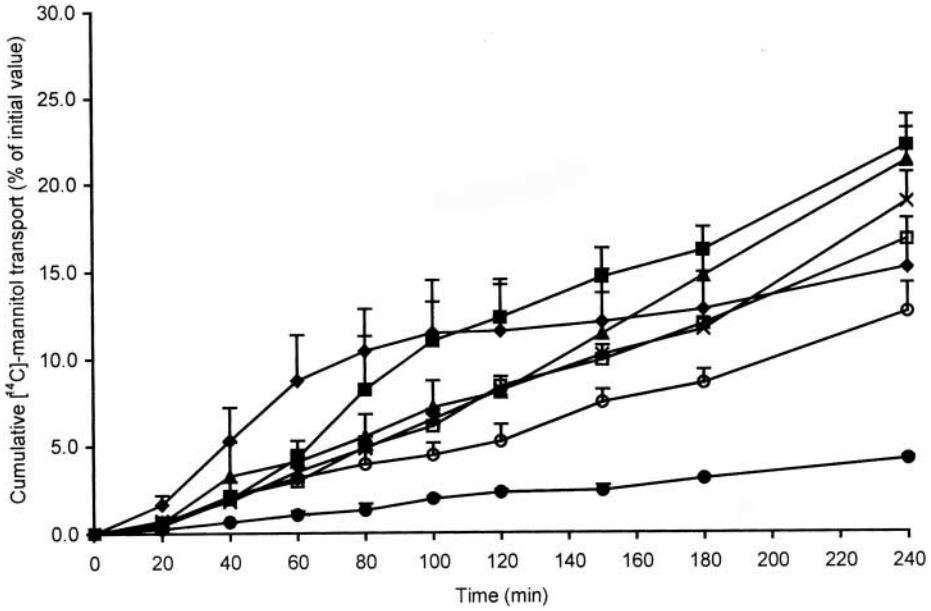

Figure 3. Cumulative transport of [^{14}C]mannitol (percentage of initial dose) across Caco-2 cell monolayers in the presence of TMC polymers (0.5% w/v) at pH 7.4. Key: control (●), TMC-12 (◆), TMC-22 (○), TMC-36 (▲), TMC-48 (■), TMC-55 (×), and TMC-59 (□). Each point represents the mean ± SD of three values.

Table 3. Cumulative transport values, apparent permeability coefficients (P_{app}) and transport enhancement ratios (R) for [^{14}C]mannitol after incubation with TMC polymers on Caco-2 monolayers at pH 7.4.

	0.25% w/v			0.5% w/v		
TMC polymer	Transport	$P_{app} \times 10^{-6}$	R	Transport	$P_{app} \times 10^{-6}$	R
Control	4.2 ± 0.1^{E}	0.62 ± 0.01	1	4.2 ± 0.1^{D}	0.62 ± 0.01	1
TMC-12	13.7 ± 1.6^{C}	1.77 ± 0.31	2.9	$15.2 \pm 2.8^{B,C}$	1.84 ± 0.29	3.0
TMC-22	8.0 ± 0.9^{D}	1.00 ± 0.19	1.6	12.6 ± 1.7^{C}	1.84 ± 0.22	3.0
TMC-36	$23.0 \pm 0.8^{A,B}$	3.19 ± 0.50	5.2	21.3 ± 2.7^{A}	3.20 ± 0.49	5.2
TMC-48	25.3 ± 1.5^{A}	3.78 ± 0.74	6.2	22.2 ± 1.0^{A}	3.53 ± 0.15	5.8
TMC-55	16.7 ± 0.6^{C}	2.53 ± 0.08	4.1	$18.9 \pm 1.7^{A,B}$	2.82 ± 0.27	4.6
TMC-59	20.7 ± 3.2^{B}	3.14 ± 0.81	5.1	$16.7 \pm 0.3^{B,C}$	2.61 ± 0.04	4.3

Each value represents the mean ± SD of three experiments. Values marked with the same letter (A, B, C, D, E) are not significantly different.

[^{14}C]mannitol obtained with TMC-36 and TMC-48 at a concentration of 0.5% w/v was in the same order, and only relatively small differences between the TMC polymers with higher degrees of quaternization were found. This indicates that the increase in the absorption-enhancing effects of TMC did not correlate up to the highest possible degree of substitution for the TMC polymers (ca. 60%), but reach a plateau already at an intermediate degree of quaternization with no further increases, even with higher degrees of quaternization.

Effect of TMC on the Transport of [^{14}C]PEG 4000

The cumulative transport of [^{14}C]PEG 4000 (percentage of initial dose) across the Caco-2 cell

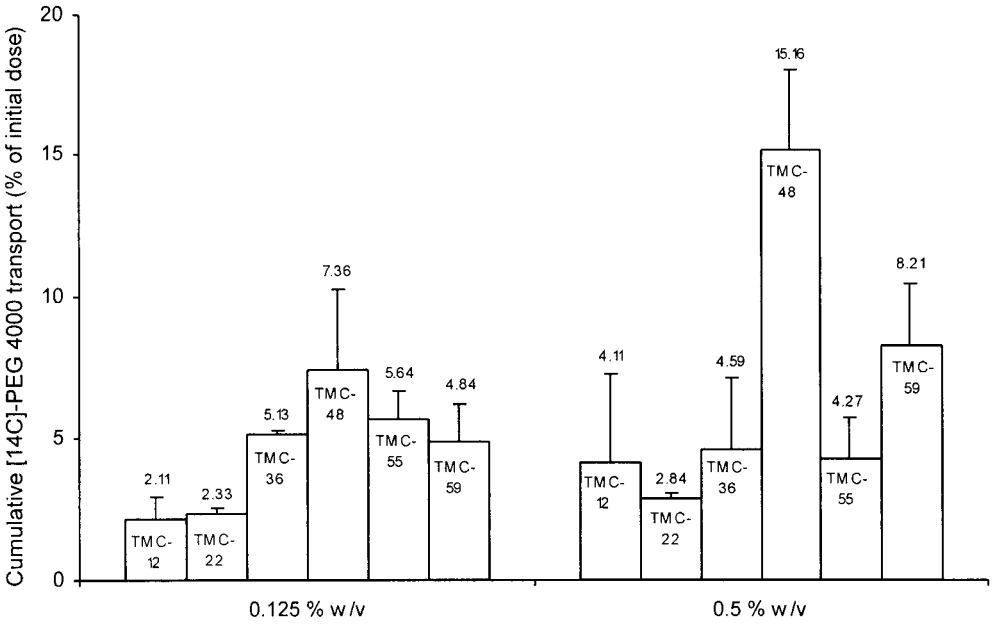

Figure 4. Cumulative transport of [^{14}C]PEG 4000 (percentage of initial dose) across Caco-2 cell monolayers 4 hr after incubation with the TMC polymers (0.5% w/v) at pH 7.4. Each point represents the mean ± SD of three values.

monolayers in the presence of the TMC polymers (0.5% w/v) at pH 7.4, 4 hr after incubation started, is shown in Fig. 4. The cumulative amount of [^{14}C]PEG 4000 (percentage of initial dose) transported across Caco-2 cell monolayers, 4 hr after incubation with the different TMC polymers (0.125 and 0.5% w/v), the calculated apparent permeability coefficients (P_{app}), and transport enhancement ratios (R) are given in Table 4.

The values in Table 4 represent a 5.6-fold (TMC-48), 3.0-fold (TMC-59), 1.7-fold (TMC-36), 1.6-fold (TMC-55), 1.5-fold (TMC-12), and 1.0-fold (TMC-22) increase in the transport at a concentration of 0.5% w/v and a 2.7-fold (TMC-48), 2.1-fold (TMC-55), 1.9-fold (TMC-36), 1.8-fold (TMC-59), 0.9-fold (TMC-22), and 0.8-fold (TMC-12) increase in the transport at a concentration of 0.125% w/v.

An apparent good correlation was found between the transport of [^{14}C]PEG 4000 across Caco-2 cell monolayers and the degree of quaternization of TMC. The transport of [^{14}C]PEG 4000 increased with an increase in the degree of quaternization of TMC until a maximum effect was reached with TMC-48, which was significantly higher compared with the transport obtained by all the other TMC polymers.

This is probably caused by optimum interactions with the negatively charged sialic groups on the membrane due to favorable chain flexibility and conformation exhibited by TMC-48 and also because of steric effects by the attached methyl groups that start to play a role at higher degrees of quaternization. Another explanation could be the saturation of the interaction mechanism with the cell membrane. As the number of positive charges increases, the number of unoccupied negative charges is reduced and the proportion of tight junctions that actually opens further declines until a maximum absolute number is reached. Further increases in the amount of positive charges will then not be associated with any increase in the number of tight junctions that are opened.

Effect of TMC on the CBF of Human Nasal Epithelia

The CBF values (percentage of initial value) of human nasal epithelial cells after incubation with the TMC polymers in a concentration of 0.5% w/v were plotted as a function of time and are shown in Fig. 5. The results show initial inhibition of the CBF of human nasal epithelial cells after incubation with the TMC polymers, but these values were maintained at the same level for the duration of the experiment and did not decrease further. This inhibitory effect

Table 4. Cumulative transport values, apparent permeability coefficients (P_{app}) and transport enhancement ratios (R) for [^{14}C]PEG 4000 after incubation with TMC polymers on Caco-2 monolayers at pH 7.4.

	0.125% w/v			0.5% w/v		
TMC polymer	Transport	$P_{app} \times 10^{-6}$	R	Transport	$P_{app} \times 10^{-6}$	R
Control	2.3 ± 0.67^{B}	0.21 ± 0.15	1	2.3 ± 0.67^{C}	0.21 ± 0.15	1
TMC-12	ND	ND	ND	$4.1 \pm 3.1^{B,C}$	0.087 ± 0.04	0.41
TMC-22	2.3 ± 0.2^{B}	0.28 ± 0.09	1.31	2.8 ± 0.2^{C}	0.43 ± 0.04	2.03
TMC-36	$5.1 \pm 0.1^{A,B}$	0.49 ± 0.27	2.33	$4.6 \pm 2.5^{B,C}$	0.73 ± 0.49	3.44
TMC-48	7.4 ± 2.8^{A}	1.03 ± 0.54	4.88	15.2 ± 2.8^{A}	1.79 ± 0.27	8.49
TMC-55	5.6 ± 1.0^{A}	0.89 ± 0.28	4.23	$4.3 \pm 1.4^{B,C}$	0.68 ± 0.31	3.24
TMC-59	$4.8 \pm 1.4^{A,B}$	0.66 ± 0.18	3.13	8.2 ± 2.2^{B}	1.18 ± 0.67	5.61

Each value represents the mean ± SD of three experiments. Values marked with the same letter (A, B, C) are not significantly different. ND, not determined.

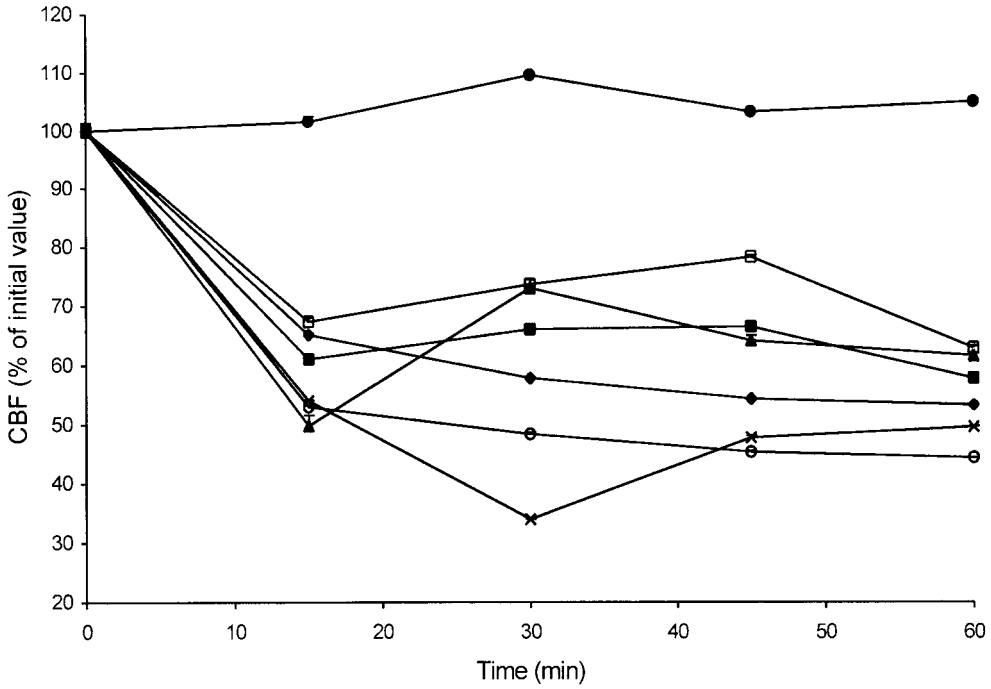

Figure 5. Effect of TMC (0.5% w/v) on the CBF of human nasal epithelial cells. Key: control (●), TMC-12 (◆), TMC-22 (○), TMC-36 (▲), TMC-48 (■), TMC-55 (×), and TMC-59 (□).

may be explained by the relatively high viscosity of the TMC polymer solutions, which result in resistance to the free movement of the cilia through the liquid phase. The TMC polymer solution could not be removed from the nasal cells without damaging the cells due to the high viscosity and mucoadhesive characteristics of these polymer solutions, and the reversibility of this effect could therefore not be measured. No apparent disruption and damaging effects could be observed on the nasal epithelial cells in contact with the TMC solutions during the CBF measurements, indicating that TMC did not induce visible local toxicity effects on the nasal epithelial cells. This is in agreement with results obtained in a

previous study conducted on the safety profile of TMC by Thanou et al.,[17] in which TMC did not lead to any nuclear staining of Caco-2 cells when incubated with the nucleic staining probe, YO-PRO®.

CONCLUSIONS

The effect of TMC polymers on the Caco-2 cell monolayers (both TEER and transport of hydrophilic and macromolecular compounds) reached a maximum value already at a degree of quaternization of 48%, and their effects did not increase further even with higher degrees of quaternization (including 55% and 59%). It is therefore concluded from the results of this study that an optimum absorption enhancement effect is already reached for TMC with an intermediate degree of quaternization (ca. 48%), and higher quaternization degrees did not significantly further increase this polymer's effects on intestinal epithelia.

A possible explanation for this phenomenon is that TMC-48 exhibits a favorable chain flexibility and conformation, and also that the attached methyl groups cause steric effects that start to play a role at higher degrees of quaternization. Another explanation could be the saturation of the electrostatic interactions between the positive charges on the TMC molecule and the negative charges on the cell membrane.

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